

AMENDMENTSIN THE SPECIFICATION:

Please replace the first paragraph of page 8 with the following:

The amino acid sequence of "pre-pro" and mature forms of rat GDNF is as set forth in Figs. 13 and 14 (SEQ ID NO:3 and residue numbers 1-134 of SEQ ID NO:4). The amino acid sequence of mature human GDNF is as set forth in the underlined portion of Fig. 19 (residue numbers 1-134 of SEQ ID NO:6). The amino acid sequence of the pre-pro form of human GDNF is set forth in Figures 19 and 22 (SEQ ID NO:28).

Please replace the third paragraph of page 8 with the following:

Also described is the cloning of the rat GDNF gene from a cDNA library prepared from the B49 cell line. The nucleic acid sequence encoding mature and pre-pro rat GDNF is set forth in Fig. 13 (SEQ ID NO:3). The method for obtaining a human gene coding for GDNF is also disclosed. The nucleic acid sequence encoding mature human GDNF is as set forth in Fig. 19 (nucleotides 105 to 506 of SEQ ID NO:5). The nucleic acid sequence encoding the first 50 amino acids of the pre-pro segment of human GDNF is as set forth in Fig. 22 (nucleotides 59 to 208 of SEQ ID NO:8).

Please replace the second paragraph of page 11 with the following:

Figure 8 (SEQ ID NO:1) describes the amino-terminal amino acid sequence obtained from purified rat GDNF. The empty parenthesis indicates a position where the amino acid could not be determined using the sequencing technique employed. Where residues are given in parentheses, there was some uncertainty as to the identification of that residue. The complete correct amino-terminal amino acid sequence is shown

in Figure 13 (residue numbers 1-25 of SEQ ID NO:4) below.

Please replace the sixth paragraph of page 11 with the following:

Figure 12 (SEQ ID NO:2) describes an internal amino acid sequence obtained from purified rat GDNF.

Please replace the eighth paragraph of page 11 with the following:

Figure 14 (residue numbers 1-134 of SEQ ID NO:4) depicts the inferred amino acid sequence of mature rat GDNF.

Please replace the second paragraph of page 13 with the following:

Figure 19 (SEQ ID NO:5) depicts a portion of the nucleic acid sequence obtained for human GDNF, as described in Example 2C below, including the entire portion of the gene encoding for mature human GDNF. Residue numbers 1-134 of SEQ ID NO:6 depicts the inferred amino acid sequence for mature human GDNF. In Figure 19, the amino acid sequence for mature human GDNF is underlined.

Please replace the second paragraph of page 14 with the following:

Figure 22 (SEQ ID NO:8) depicts a portion of the nucleic acid sequence obtained for human GDNF, as described in Example 2D below, including the coding sequence of amino acids 1-50 of human pre-pro GDNF. Also depicted is the inferred amino acid sequence for the first 50 amino acids of human pre-pro GDNF (SEQ ID NO:29). This information, in conjunction with coding sequence information given in Figure 19, provides the full coding sequence for human pre-pro GDNF (nucleotides 59 to 691 of SEQ ID NO:25), and the inferred amino acid sequence for the human pre-pro GDNF protein (SEQ ID NO:28).

Please replace the second paragraph of page 26 with the following:

The inferred amino acid sequence given in Figure 14 (residue numbers 1-134 of SEQ ID NO:4) shows the amino acid sequence for the "mature GDNF". By "mature GDNF", is meant the sequence of the purified GDNF obtained from the B49 conditioned medium. Of course, the purified GDNF may exist as a dimer or other multimer and may be glycosylated or chemically modified in other ways. Mature GDNF may be truncated at the carboxyl terminus, in particular by proteolytic processing of the Lys-Arg residues 6 and 5 residues from the carboxyl terminal end. Examination of the nucleic acid sequence of the 1ZapII76.1 rat clone as shown in Fig. 13 (SEQ ID NO:3) suggests that GDNF is initially translated as a pre-pro-GDNF polypeptide and that proteolytic processing of the signal sequence and the "pro" portion of this molecule result in purified GDNF having the same mature sequence as that obtained from B49 conditioned medium. It is postulated, that the pre-pro GDNF polypeptide begins at the first ATG --methionine encoding -- codon at the 5' end of the clone (position 50 in Figure 13). The present invention includes, therefore, any and all pre-pro GDNF polypeptides that may be translated from the gene shown in Figure 13, as well as any and all pre-pro GDNF polypeptides translated from a more complete clone that may be easily obtained by one of skill in the art using standard laboratory procedures and the clone described herein.

Please replace the first and second paragraph of page 27 with the following:

Review of the rat nucleic acid sequence given in Fig. 13 (SEQ ID NO:3) shows that the predicted amino acid sequence located between positions 541 and 561 is Asp-Lys-Ile-Leu-Lys-Asn-Leu which is consistent with the amino acid sequence determined for a peptide derived from purified mature rat GDNF

by the process described in the section on internal sequence in Example 1 below. A TGA stop codon at positions 706-708 of SEQ ID NO:3 terminates the ORF. The predicted length of the purified GDNF is thus 134 amino acid residues, and the predicted molecular weight of this polypeptide is 14,931. Two potential N-linked glycosylation sites occur at [positions 425 and 533] the Asn and Tyr at residue 49 and 57 of SEQ ID NO:3, respectively. Glycosylation at either or both of these sites would increase the molecular weight of the molecule.

The serine residue encoded by nucleotides 304-306 of SEQ ID NO:3, which corresponds to the start of the sequence of purified mature GDNF, is preceded by the sequence Lys-Arg which provides a potential proteolytic cleavage site for processing of a putative precursor form of GDNF to produce the form of the molecule that is purified from B49 cells. A potential translational initiation codon (ATG) occurs at positions 73-75 of SEQ ID NO:3 and is closely followed by a potential secretory signal sequence. The sequences flanking this ATG show sufficient similarity to the Kozak consensus sequence (Kozak 1987 Nucleic Acids Res. 15:125-48) to indicate that this ATG could be utilized as a translational initiation site. Moreover, this ATG is the most 5' ATG in the sequence of the cDNA clone. These facts suggest it as a potential start site for translation of a precursor form of GDNF.

Please replace the first paragraph of page 28 with the following:

These above noted features of the nucleotide sequence of the rat cDNA clone suggest the possibility that GDNF is initially translated as a pre-pro GDNF polypeptide and that proteolytic processing of the signal sequence and the "pro" portion of this molecule result in production of the form of GDNF that is purified from B49 cell conditioned medium. However, the occurrence of other forms of GDNF is also consistent with the sequence data. For example, two other potential ATG translational starts occur within the 681 bp

ORF: one at residue -25 and one at residue -13 as shown in SEQ ID NO:3. These ATG's are located upstream of the start of the amino-terminal sequence of purified GDNF. Although, in eukaryotes, translational initiation generally occurs at the 5'-most ATG of the mRNA (Kozak, supra,) there are instances in which a proportion of the translational initiations occur at a downstream ATG. Thus, alternative precursor forms of GDNF could conceivably arise by translational initiation at these ATG codons. Proteolytic processing of these polypeptides could result in production of the same form of purified GDNF observed in B49 cell conditioned medium. Moreover, the open reading frame extends through the 5' end of the sequence of the cDNA clone. It is therefore possible that the initiation of translation occurs at an upstream ATG not present in the cDNA clone. In this eventuality, GDNF would be translated as an even larger precursor containing the amino acid sequence described here and additional sequence upstream. Processing of such a hypothetical precursor form could also lead to production of the purified form of GDNF reported here. It would be possible to detect potential upstream ATG starts by sequencing the 5' end of the mRNA containing the GDNF gene via primer extension with reverse transcriptase (Maniatis et al. supra). Additionally, other cDNA clones could be obtained from B49 libraries and the 5' ends of these clones could be sequenced. The size of the 5' mRNA located upstream of the first ATG could be more roughly determined by the techniques of "S1 mapping" (Maniatis et al. supra) and/or simple sizing of primer extension products of the reverse transcriptase reaction. While a variety of putative forms of the primary translational product that contains the sequences encoding purified GDNF can be postulated, the partial DNA sequence presented here for the cDNA clone carried in the recombinant phage 1ZapII76.1 clearly defines the coding sequence that constitutes the purified GDNF polypeptide isolated from the B49 cell conditioned medium.

Please replace the first paragraph of page 30 with the following:

Specific nucleic acid sequences can be modified by those of skill in the art. Therefore, this invention also includes all nucleic acid sequences which encode for the amino acid sequences for mature rat and mature human GDNF as set forth in Figures 14 (residue numbers 1-134 of SEQ ID NO:4) and 19 (residue numbers 1-134 of SEQ ID NO:6), and pre-pro rat GDNF as set forth in Figure 13 (SEQ ID NO:3) and for pre-pro human GDNF as set forth in Figures 19 and 22 (SEQ ID NO:28). The present invention also incorporates nucleic acid sequences which will hybridize with all such nucleic acid sequences -- or the complements of the nucleic acid sequences where appropriate -- and encode for a polypeptide having dopaminergic activity. The present invention also includes nucleic acid sequences which encode for polypeptides that have dopaminergic activity and that are recognized by antibodies that bind to GDNF.

Please replace the first and second paragraph of page 66 with the following:

Five of the six λ clones gave identical hybridization patterns. Probe 1 hybridized to an approximately 700 bp Eco R1 fragment and probe 2 hybridized to an approximately 2.8 Kb Eco R1 fragment. The fact that the two probes hybridized to two different Eco R1 DNA fragments strongly suggested that the human GDNF gene contains an homologous Eco R1 site. The 700 bp and the 1.8 Kb Eco R1 fragments were subcloned separately into Bluescript SK- (Stratagene). Nucleotide sequences of these two fragments were determined as described in Example 2B. The sequence of these DNA fragments is shown in Figure 19 (SEQ ID NO:5). From the sequence it is clear that there is an intron preceding amino acid 52 of pre-pro GDNF. There is no intron in the portion of the gene that codes for mature human GDNF. The predicted amino acid sequence of mature human GDNF is 93%

homologous to mature rat GDNF. This is approximately the same degree of amino acid sequence homology found among rat and human proteins for other neurotrophic factors (Amino acid sequence homology between rat and human CNTF is 83%; McDonald et al. BBA (1991) (in press). Amino acid sequence homology between rat and human NGF is 95%, BDNF is 100%, and NT-3 is 100%; Hallbook et al. 1991 Neuron 6:845-855).

To obtain the complete human pre-pro GDNF sequence, a radiolabeled hybridization probe may be made based on the sequence of human GDNF already obtained and use this to screen human cDNA libraries. Because cDNAs are copies of the processed mRNA, the introns are not present and the sequence of the complete coding sequence can be obtained. Alternatively, now that the position of the intron relative to the coding sequence is known, a hybridization probe that is specific for sequences upstream of the intron can be made from the rat cDNA clone and this probe can be used to screen a genomic library for clones that contain the 5' exon(s).

Please replace the first and second paragraph of page 67 with the following:

D. Nucleotide Sequence Encoding the First 50 Amino Acids of Human Pre-pro GDNF

As detailed in Example 2C, there is an intron that splits the nucleotide sequence corresponding to amino acid 51 of human pre-pro GDNF (i.e., between the T and CA forming the codon for the serine residue at position 51). In order to obtain the sequence of this portion of the molecule, a human genomic library was screened with a probe derived from the amino-terminal coding sequence of rat pre-pro GDNF and one hybridizing clone was sequenced and shown to contain the coding sequence of amino acids 1 through 50 of human pre-pro GDNF as shown in Figure 22 (SEQ ID NO:8). SEQ ID NO:25 (nucleotide 59 to 691) and SEQ ID NO:28 present nucleotide and amino acid sequences, respectively, for a composite pre-pro sequence as depicted in Figures 22 and 19 as well SEQ ID NOS:8

and 5. A pre-pro form of human glial cell line-derived neurotrophic factor polypeptide is set forth in SEQ ID NO:26 (amino acid residues -77 through 134).

For this library screen a PCR probe was synthesized as described in Example 2C. The oligonucleotide primers employed were:

PD1 = 5' > CCCGAATTCGACGGGACTCTAAGATG > 3' (SEQ ID NO:19)

LFA = 5' > CGGTGGCCGAGGGAGTGGTCTTC > 3' (SEQ ID NO:20)

Conditions for the PCR (both "cold" and ³²P-labelling) reactions were as described in Example 2B except that the reaction consisted of 25 or 30 cycles of: 95°C for 1 min., 50°C for 1.5 min., and 72°C for 1 min. The product of this reaction contains the first 122 bp of rat pre-pro_GDNF coding sequence and 14 base pair 5' to the putative initiator ATG (see Figure 13 and SEQ ID NO:3). Conditions for screening the human genomic library with this probe were as described in Example 2C. The same filter lifts used to identify clones carrying sequences for mature human GDNF were washed twice for 15 min. in deionized-distilled H₂O heated to boiling, probed overnight, and washed according to the protocol described in Example 2C. The filters were exposed to film for 3 days at -70°C with intensifying screens. When developed, numerous duplicate positives of varying intensities were observed. Twelve relatively strong positives were picked and 10 of these were plaque purified by successive rounds of hybridization under the screening conditions.

Please replace the first and second paragraph of page 69 with the following:

Figure 22 (SEQ ID NO:8) presents 233 base pairs of the sequence thus obtained. This sequence contains a region of 151 bp that exhibits a very high degree of homology with the first 151 bp of coding sequence for rat pre-proGDNF; 88% identity at the amino acid level and 95% identity at the DNA level. Therefore, it is concluded that this region is part of the exon that carries the coding sequence of the amino-terminal 50

residues of the human pre-pro GDNF and the first nucleotide of the codon for residue 51 (nucleotide 209 of SEQ ID NO:25). The sequence immediately 3' to this 151 bp sequence is homologous to the consensus sequence for the 5' end of mammalian introns [Shapiro and Senapathy 1987 Nucl. Acids Res. 15:7155-7174]. The sequence immediately 5' to the putative initiator ATG shows strong homology to the rat sequence for 28 bp; 27 of 28 residues are identical. At this point the upstream sequence diverges sharply. The sequence around the point of divergence shows considerable homology to the consensus sequence for the 3' end of mammalian introns. (Shapiro and Senapathy, supra.) Thus, it seems likely that this is a splice site although there is no direct evidence for this. The open reading frame containing human pre-pro GDNF extends at least 27 base pairs upstream of the initiator ATG. As discussed in the Detailed Description of Preferred Embodiments above, it is possible that other forms of a pre-pro GDNF could be produced that would contain additional upstream amino acids. These forms might also be processed to produce the mature GDNF molecule that has been purified and sequenced (see Example 1).

The nucleotide sequences presented here and in Example 2C, contain the entire coding sequence for a human pre-pro GDNF (SEQ ID NO:28) that exhibits extensive homology to the rat pre-pro GDNF (SEQ ID NO:27) which has been successfully expressed in mammalian cells (see Example 5) to produce active rat GDNF.

Please replace the existing Sequence Listing found on pages 92 - 99D of the Specification with pages 1-19 of the revised Sequence Listing enclosed herewith.